

# Living Systems Develop ever more Complex Processes and Acquire new Features

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## Description

Engineering new functionality into living eukaryotic systems by enzyme evolution or *de novo* protein design is a formidable challenge. Cells do not rely exclusively on DNA-based evolution to generate new functionality but often utilize membrane encapsulation or formation of membraneless organelles to separate distinct molecular processes that execute complex operations. Applying this principle and the concept of two-dimensional phase separation, we develop film-like synthetic organelles that support protein translation on the surfaces of various cellular membranes. These sub-resolution synthetic films provide a path to make functionally distinct enzymes within the same cell. We use these film-like organelles to equip eukaryotic cells with dual orthogonal expanded genetic codes that enable the specific reprogramming of distinct translational machineries with single-residue precision. The ability to spatially tune the output of translation within tens of nanometers is not only important for synthetic biology but has implications for understanding the function of membrane-associated protein condensation in cells.

## Living Systems Develop

How living systems develop ever more complex processes and acquire new features is not only a central question in evolutionary biology but is also an essential consideration for synthetic biologists striving to create new and complex functionalities in a living host. A common evolutionary mechanism is the duplication of genes or even whole genomes. This creates redundant biomolecules, which can undergo divergence, giving rise to new functions. Intriguingly, duplicated genes are often deleted, and recent studies have shown that paralogous genes can more readily diverge if their functional and structural entanglement is comparatively weak. Thus, to develop new functionalities, it often seems necessary that genes become orthogonal and do not cross-react with ancestral biomolecules. For synthetic biology applications in cells, such orthogonality is generally essential to prevent interference with the endogenous processes of the host, and it can be a daunting task to develop this *de novo* for molecules that have many

interaction partners. Furthermore, creating enzymes that specifically execute desired functions *de novo* is challenging. Therefore, alternative strategies for generating orthogonal enzymes inside a cell would be extremely useful.

## Genetic Code Expansion

One powerful tool that synthetic biologists have for creating new functions *in vivo* is genetic code expansion (GCE), which has been widely used to site-specifically incorporate noncanonical amino acids (ncAAs) into proteins *in vivo*. The genetic code determines how genomic information is transferred into a polypeptide sequence through the central dogma and relies on aminoacyl-tRNA synthetase (aaRS)/tRNA pairs to decode triplet codons into specific amino acids. These pairs have extensive protein-RNA and, in the case of multimeric aaRS systems, protein-protein interaction surfaces. Engineering functional derivatives is thus a formidable challenge. Hence, to repurpose aaRS/tRNA pairs to encode noncanonical functionalities in a particular host, aaRS/tRNA pairs from highly evolutionarily distinct organisms are typically used, a few of which are orthogonal to the new host machinery. Here, orthogonal refers to a given aaRS accepting only a specific ncAA, and then only aminoacylating it to its cognate tRNA. In addition, the cognate tRNA should not be recognized as a substrate by any of the endogenous tRNA synthetases.

For the simultaneous incorporation of multiple different ncAAs in eukaryotes, GCE technology is fundamentally limited by three problems: (1) the translational process lacks mRNA specificity such that other mRNAs in the transcriptome that naturally terminate at amber codons can be mistranslated; (2) the number of codons that can be reassigned without altering host functionality is limited; and (3) the dearth of orthogonal aaRS/tRNA pairs.

We recently published a solution to the first of these problems: Membraneless Orthogonally Translating (OT) organelles that are formed by phase separation and targeting to microtubule plus-ends to afford a micron-sized organelle. We define the term organelle as a spatially distinct site in the cell, regardless of its

structure or appearance, which nevertheless executes a specific function and has a composition distinct from its surroundings. Phase separation occurs at above the critical concentrations of certain proteins that were fused to PylRS and the ms2 bacteriophage coat protein (MCP), an RNA-binding protein. Although the suppressor tRNA itself is a relatively small molecule, the PylRS-loaded organelle efficiently recruits it, leading to a very high concentration inside the condensate and a very low concentration throughout the rest of the cell. The POI mRNA is labeled in the 3' untranslated region with specific RNA motifs (ms2 loops) that are bound by MCP, thus leading to recruitment of the mRNA into the organelle. Because only the ribosomes processing the recruited mRNA are exposed to a very high concentration of suppressor tRNAPyl, it is preferentially translated according to an expanded genetic code. By contrast, ribosomes translating mRNA elsewhere in the cell terminate translation once the chosen stop codon is encountered, because no tRNAPyl is available. Note that the translational machinery requires that a few hundred factors work smoothly together and no component other than the PylRS and MCP were fused to the organelle scaffold.

Thus, despite sharing all other components of translation with the cytoplasm, from which these components are essentially freely accessible, we detected up to 8-fold selectivity for amber suppression of targeted (i.e., ms2 tagged) versus untargeted mRNAs. We also showed that the same logic could be applied to reprogramming the opal or ochre codon. Hereafter, we refer to this particular OT organelle-based GCE technology as being mRNA selective.

We thus developed several OT film-like organelles, which enabled us to design multiple spatially orthogonal aaRS/tRNA pairs within the same cytoplasm. This allowed us to reuse the same stop codon to incorporate distinct ncAAs into different proteins *in vivo*, effectively generating a cell with three spatially and functionally distinct translational programs. We further discuss the implications of these results for membrane signaling and membrane-associated phase separation.